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# Cataloging altered gene expression in young and senescent cells using enhanced differential display

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## ABSTRACT

Recently, a novel PCR-based technique, differential display (DD), has facilitated the study of differentially expressed genes at the mRNA level. We report here an improved version of DD, which we call Enhanced Differential Display (EDD). We have modified the technique to enhance reproducibility and to facilitate sequencing and cloning. Using EDD, we have generated and verified a catalog of genes that are differentially expressed between young and senescent human diploid fibroblasts (HDF). From 168 genetags that were identified initially, 84 could be sequenced directly from PCR amplified bands. These sequences represent 27 known genes and 37 novel genes. By Northern blot analysis we have confirmed the differential expression of a total of 23 genes (12 known, 11 novel), while 19 (seven known, 12 novel) did not show differential expression. Several of the known genes were previously observed by others to be differentially expressed between young and senescent fibroblasts, thereby validating the technique.

## INTRODUCTION

The introduction of the differential display (DD) technique (1) provided a method to study differential gene expression. DD detects expressed mRNAs by PCR amplification of DNA fragments that represent the 3' end of an mRNA population.

The reproducibility of the original DD technique has been low and thus many of the initially identified genetags were false positives (2–4). Several papers, including those from the original authors (2), have suggested improvements for DD. Adjustments have been made in the design of the primers (2,5–8), in the choice of reagents (6,8–10) and improvements have also been reported in the processing and analysis of the bands (11–13).

While initiating a senescent gene expression research project, we designed improvements in DD to enhance its reproducibility. Our strategy was to introduce a two step PCR amplification, in which the first four PCR cycles are done at low annealing temperatures, while efficient amplification is carried out in subsequent PCR cycles at high annealing temperatures. Moreover, we have simplified the subsequent cloning and sequencing of bands.

Using longer primers and this two-step PCR protocol, we demonstrate a reproducible DD technique that we call enhanced differential display (EDD). Several characteristics EDD are analyzed in this paper and EDD is used to catalogue differences between young and senescent human diploid fibroblasts (HDFs).

The *in vitro* culture of normal HDFs has long served as a model system for studying replicative senescence (14). Human diploid fibroblasts, like other normal somatic cells, have a finite replicative capacity (15,16). The *ex vivo* proliferative potential of HDFs is inversely related to the age of the donor (17–19) supporting the notion that replicative senescence is a genetically determined series of changes exhibited by normal cells that culminates in exit from the cell cycle. Senescent cells change their pattern of gene expression and exhibit an altered phenotype (20–27). However, most senescent cells remain viable and are metabolically active (28,29). This altered gene expression in replicative senescent cells may play a role in the pathogenesis of age-related disease (25,30,31).

A number of genes that are differentially expressed during senescence have been reported in the literature (21,25,27,28). However, these studies have used different cell types and growth conditions. It was therefore not feasible to derive a catalog of genes with altered expression during senescence from such studies that reflected one standard growth condition. Moreover, subtractive hybridization techniques (26,32) suggest that many differentially regulated genes in replicative senescence have not yet been identified. Thus, we expected that many novel genes might be identified by developing a comprehensive EDD catalog of genes expressed differentially in young and replicative senescent cells under standard growth conditions. Our catalog demonstrates the value of EDD by identifying 12 known genes and 11 novel genetags that are differentially regulated during replicative senescence.

## MATERIALS AND METHODS

### Cell culture and RNA preparation

Human fibroblasts strains, derived from neonatal foreskin (BJ) and fetal lung (IMR90), were a kind gift from Dr Jerry Shay (University of Texas, Southwestern Medical Center at Dallas, TX). Fibroblasts were split prior to confluency according to standard tissue culture techniques. Cells were grown in DMEM

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medium plus 10% Bovine Calf Serum (BCS). The last split before RNA isolation was 1–8 for young cells and 1–2 for senescent cells, after which two protocols were followed: (i) cells were grown at 37°C in DMEM medium plus 10% BCS for 2 days; then RNA was isolated (see below); (ii) to induced quiescence, cells were first seeded in DMEM medium plus 10% BCS to allow attachment (4–8 h). The medium was then replaced with DMEM medium plus 0.5% BCS and the cells were grown for 3 days at 37°C. The medium was changed with fresh DMEM medium plus 0.5% BCS and the cells were grown for an additional 2 days at 37°C, after which RNA was isolated.

For RNA isolation, cells were quickly washed once with PBS and then collected in GITC solution [4 M guanidine isothiocyanate, 25 mM sodium acetate (pH 6) and 0.83% beta-mercaptoethanol]. Following a standard protocol (33), 7 ml of the lysis solution were layered on a CsCl cushion (5.7 M in 25 mM sodium acetate, pH 6) and then centrifuged at 32 000 r.p.m. for 20 h at 20°C in a SW41 rotor. After carefully removing the supernatant, the RNA pellets were resuspended in diethylpyrocarbamate-treated deionized H<sub>2</sub>O (DEPC-water), then precipitated by adding 1/10 vol of 3 M sodium acetate, pH 6 (filter-sterilized, made with DEPC-water) and 2.5 vol of cold 100% ethanol. The precipitate was collected by centrifugation and the RNA pellet was dissolved in DEPC treated H<sub>2</sub>O and stored at –70°C.

### Primers

The primers used in this study are listed in Table 1. Arbitrary primers were designed to have a GC content of 50% and were inspected for the absence of obvious hairpin structures. Some of the 3' sequence parts of the primers (10,14,34–38) were derived from the sequences listed by Bauer *et al.* (5), who used similar criteria for primer design.

### Synthesis of first-strand cDNA

One microgram of total RNA was mixed with 2.5 µl of 20 µM 3'-primer and 9.5 µl of DEPC-H<sub>2</sub>O. The resulting solution was placed at 75°C for 10 min and then chilled quickly. Next was added 5 µl of 5× first strand synthesis buffer; 1 µl RNAsin (Pharmacia); 2.5 µl of 0.1 mM DTT; 2.5 µl of 0.25 mM dNTP and 1 µl of reverse transcriptase (SuperScript™ II RT, BRL) and the resulting solution was incubated at 37°C for 70 min. The reaction was terminated by incubating the mixture at 95°C for 10 min, then stored at –20°C.

### PCR amplification of cDNA

The reaction mixture was prepared using 1 µl of the cDNA reaction (3' primer carried over from cDNA), 2 µl of 10 × PCR buffer (500 mM KCl, 100 mM Tris pH 8.3, 20 mM MgCl<sub>2</sub>), 1.5 µl of 0.1 mM dNTP, 1.25 µl of 20 µM 5' primer, 1 µl of a 1 to 5 dilution of [ $\alpha$ -<sup>32</sup>P]dATP (800 µCi/µM, NEN Dupont), 0.5 µl of *Taq* polymerase (Boehringer Mannheim) and 12.75 µl DEPC-H<sub>2</sub>O. Mineral oil was layered on top of the reaction mixture, which was then centrifuged briefly to collect the reaction mixture at the bottom of the tube. The PCR was performed using a thermocycler (Perkin-Elmer model) programmed to conduct four cycles (94°C for 45 s; 41°C for 60 s; and 72°C for 60 s) and then 18 cycles (94°C for 45 s; 60°C for 45 s; and 72°C for 120 s). The tubes were centrifuged briefly to collect the reaction mixture at the bottom and can be stored at 4°C. Reaction products were

denatured in formamide sequencing dye and then resolved on standard 6% polyacrylamide, 7 M urea, sequencing gels. Gels were dried onto Whatman 3 M paper and exposed to Kodak XAR5 film overnight.

### Removing differentially displayed bands

The film and gel were lined up by three asymmetric needle holes. Bands were marked, excised from the gel and placed into a 1.5 ml tube with 1 ml of TE buffer. After 15 min the paper and TE were removed and 1 ml of (TE + 100 mM NaCl) was added to the polyacrylamide slice. The tube was placed in a boiling water bath for 10 min and then left at room temperature overnight. Five microliters from this tube was used for PCR amplification. The PCR (5 µl 10 × PCR buffer, 2.5 µl 1 mM dNTP, 3 µl 20 µM 5'-primer, 3 µl 20 µM 3'-primer, 1 µl *Taq* polymerase, 5 µl sample and DEPC water to 50 µl final volume) was carried out for 25 cycles (94°C for 45 s; 60°C for 60 s; and 72°C for 60 s).

**Table 1.** Sequence of the primers used in EDD (see text for further details)

Primers used in EDD for human fibroblasts	
3'-(T-rich)-primers:	
A:	5'-GCG CAA GCT TTT TTT TTT TTC T-3'
B:	5'-GCG CAA GCT TTT TTT TTT TTC C-3'
C:	5'-GCG CAA GCT TTT TTT TTT TTC G-3'
D:	5'-GCG CAA GCT TTT TTT TTT TTG T-3'
E:	5'-GCG CAA GCT TTT TTT TTT TTG G-3'
F:	5'-GCG CAA GCT TTT TTT TTT TTG A-3'
G:	5'-GCG CAA GCT TTT TTT TTT TTA T-3'
H:	5'-GCG CAA GCT TTT TTT TTT TTA C-3'
J:	5'-GCG CAA GCT TTT TTT TTT TTA G-3'
K:	5'-GCG CAA GCT TTT TTT TTT TTA A-3'
L:	5'-GCG CAA GCT TTT TTT TTT TTC A-3'
M:	5'-GCG CAA GCT TTT TTT TTT TTG C-3'
5'-arbitrary-primers:	
00:	5'-CGG GAA GCT TAT CGA CTC CAA G-3'
01:	5'-CGG GAA GCT TTA GCT AGC ATG G-3'
02:	5'-CGG GAA GCT TGC TAA GAC TAG C-3'
03:	5'-CGG GAA GCT TTG CAG TGT GTG A-3'
04:	5'-CGG GAA GCT TGT GAC CAT TGC A-3'
05:	5'-CGG GAA GCT TGT CTG CTA GGT A-3'
06:	5'-CGG GAA GCT TGC ATG GTA GTC T-3'
07:	5'-CGG GAA GCT TGT GTT GCA CCA T-3'
08:	5'-CGG GAA GCT TAG ACG CTA GTG T-3'
09:	5'-CGG GAA GCT TTA GCT AGC AGA C-3'
10:	5'-CGG GAA GCT TCA TGA TGC TAC C-3'
11:	5'-CGG GAA GCT TAC TCC ATG ACT C-3'
12:	5'-CGG GAA GCT TAT TAC AAC GAG G-3'
13:	5'-CGG GAA GCT TAT TGG ATT GGT C-3'
14:	5'-CGG GAA GCT TAT CTT TCT ACC C-3'
15:	5'-CGG GAA GCT TAT TTT TGG CTC C-3'
16:	5'-CGG GAA GCT TTA TCG ATA CAG G-3'
17:	5'-CGG GAA GCT TTA TGG TAA AGG G-3'
18:	5'-CGG GAA GCT TTA TCG GTC ATA G-3'
19:	5'-CGG GAA GCT TTA GGT ACT AAG G-3'

### Direct PCR sequencing differentially displayed bands

Amplified bands were separated from the primers on a low melting agarose gel and the bands were excised. Each gel slice

was washed twice with TE, melted at 60°C and 5 µl were taken for direct sequencing using the PCR cyclist kit (Stratagene) with the appropriate 5' primer that was used to generate the band. Typically, 30–40 bases of unambiguous DNA sequence could be read and were compared to the Genbank and EMBL databases (BLAST network service NCBI).

### Cloning differentially-displayed bands

The PCR-amplified bands were digested with *Hind*III and then run on a 2% low melting agarose gel. The band was excised, then melted at 65°C. Ligation reactions were performed using 2 µl of DNA in melted agarose and 1 µl of phosphatase-treated, *Hind*III-digested pBluescript (Stratagene; ~0.2 µg/ml), 2 µl of 10 mM ATP, 2 µl of 10 × One Phor-All<sup>TM</sup> buffer (Pharmacia), 12 µl of dH<sub>2</sub>O and 1 µl of T4 DNA ligase (Pharmacia) in a total volume of 20 µl. The reaction was incubated at 37°C for 2–3 h and then used to transform competent *Escherichia coli* DH5α (BRL). Transformants were analyzed by sequencing and plasmid DNA was prepared using standard plasmid preparation techniques (39).

### Northern and Southern blot analysis

Northern and Southern blot analysis was performed according to standard procedures (39). Nucleic acids were transferred to charged nylon membranes, then cross-linked using a UV Stratalinker (Stratagene). Probes for novel gene tags were prepared by restriction digestion of the appropriate plasmid with *Hind*III followed by isolation of the insert band on low-melting agarose and then radioactive labeling of the DNA using the random hexamer-primed method (40). Following hybridization to the probe, the filter blot was washed twice in 1× SSC and 0.5% SDS at 65°C for 30 min. The blots were then exposed and analyzed using a PhosphorImager 425E (Molecular Dynamics). Probes for known genes are prepared by kinase end labeling of 40mer oligos that were designed to be complementary to the 5' coding region of the message. The following probes were used in this study: 80K-L protein, human, GenBank accession number (GBAN): D10522, bases: 497–458, 5'-CCG TTT ACC TTC ACG TGG CCA TTC TCC TGT CCG TTC GCT T-3'; aldehyde dehydrogenase 1, human, GBAN: K03000, bases: 186–147, 5'-AGG AAC AAT ATT CAC TAC TCC AGG AGG AAA CCC TGC CTC T-3'; cell adhesion molecule (CD44), human, GBAN: M59040, bases: 166–127, 5'-CCG AGA GAT GCT GTA GCG ACC ATT TTT CTC CAC GTG GTA T-3'; collagenase, human, GBAN: X05231, bases: 181–142, 5'-CCA GGT ATT TCT GGA CTA AGT CCA CAT CTT GCT CTT GTG T-3'; collagen 1 alpha 1, human, GBAN: K01228, bases: 990–951, 5'-ATC AGC ACC TTT GGG ACC AGC ATC ACC TCT GTC ACC CTT A-3'; collagen 1 alpha 2, human, GBAN: J03464, bases: 519–481, 5'-AAG GTT ACT GCA AGC AGC AAC AAA GTC CGC GTA TCC ACA A-3'; collagen 3 alpha 1, human, GBAN: X14420, bases: 142–103, 5'-CGA GAA GTA GCC AGC TCC CCT TTT GCA CAA AGC TCA TCA T-3'; elastin, human, GBAN: M36860, bases: 183–144, 5'-CCT GGA TAA AAG ACT CCT CCA GGA ACT CCA CCA GGA ATG G-3'; EPC-1/PEDF, GBAN: M76979, bases: 256–217, 5'-AAG AAA GGA TCC TCC TCC TCC ACC AGC GCC CCT GTG CTG T-3'; heregulin-beta 2, human, GBAN: M94167, bases: 654–615, 5'-GAG GAG TAT TCA GAA CTG GTT TCA CAC CGA AGG ACT AGT T-3'; human tissue factor, GBAN: M16553, bases:

357–318, 5'-CTC GTC GGT GAG GTC ACA CTC TGT GTC TGT TGT GTA AAA C-3'; IGF binding protein 5, human, GBAN: M62403, bases: 538–499, 5'-CTG GTG CTC CGG TCT CGA ATT TTG GCG AAG TGC TTC TGC A-3'; laminin A, human, GBAN: X70904, bases: 193–154, 5'-CTC CAT ATT GAT AGG CGT GCT CTA TTG CTC TAG GGC TGT T-3'; PAI-1, human, GBAN: M16006, bases: 340–301, 5'-TCT TGA ATC CCA TAG CTG CTT GAA TCT GCT GCT GGG TTT C-3'; PAI-2, human, GBAN: M18082, bases: 74–34, 5'-ATT GAG GGC AAA GAG TGT GTT TGC CAC ACA AAG ATC CTC C-3'; TPA, human, GBAN: X13097, bases: 299–259, 5'-TTG CTT CTG AGC ACA GGG CGC AGC CAT GAC TGA TGT TGC TG-3'; UPA, human, GBAN: K03226, bases: 405–366, 5'-ATC TGT GGG CAT GGT ACG TTT GCT GAA GGA CAG TGG CAG A-3'.

## RESULTS

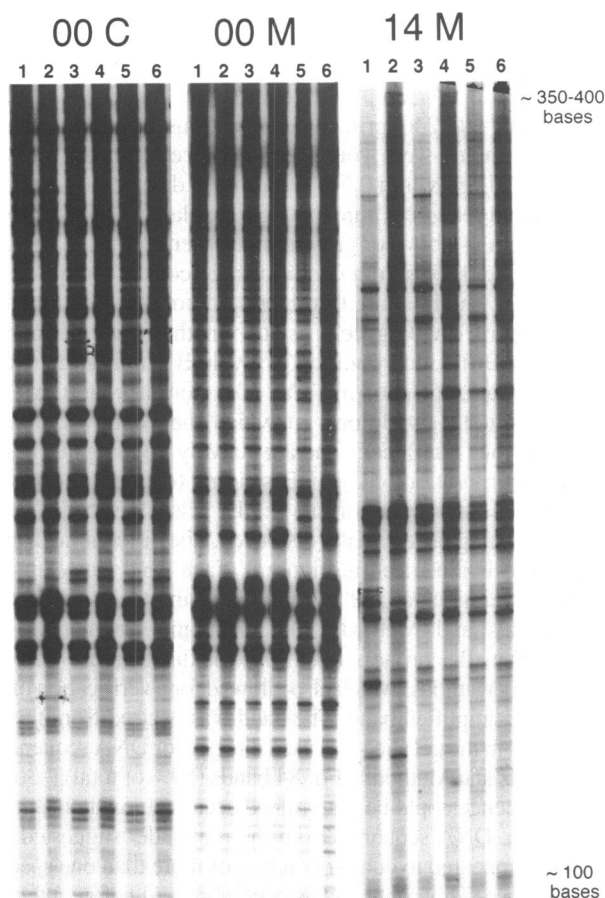
### Enhanced differential display

Using the original DD technique, we experienced inconsistency in the banding patterns between repeat experiments. We suspected that the high number of low-stringency PCR cycles in the original protocol [40 cycles with a 40°C annealing temperature (41)] might contribute to this inconsistency. To allow for increased stringency, we increased the length of the primers to 22 nucleotides and then used low annealing temperatures (41°C) for only the initial few PCR cycles. Under these conditions, the primers would behave in a similar way to the short 10 nucleotide primers of the original DD technique; they serve to prime synthesis from sequences that are complementary to the 3' end of the primer. For the remaining PCR cycles, the annealing temperature was raised during which the longer length of the primers would allow for efficient replication of products made in the initial cycles. The result was a significant improvement in the reproducibility of banding patterns, since now the majority of the PCR was performed under stringent conditions. A range of temperature profiles and cycle numbers was sampled and the final protocol used in this study consists of four initial cycles with a 41°C annealing step followed by 18 cycles with an annealing temperature of 60°C (see Materials and Methods). The longer primers also allow direct PCR sequencing of a PCR band after amplification and gel purification, while the presence of a restriction site, *Hind*III, facilitates rapid cloning. Others have also reported improvements in DD using altered primer design, cycle temperatures and cycle conditions (8).

Three typical results of such EDD reactions are shown in Figure 1, in which six samples of HDFs in various growth conditions are compared using three primer sets. Experiments with the same primer sets have been repeated several times, with similar results. Although the two strains of fibroblasts were derived from different tissues, the expression patterns for both strains are remarkably similar. We have observed bands that are specific for BJ or IMR90, irrespective of replicative senescence, occurring with a frequency of ~2% (one band per primer set).

### Specificity of 5' primer in EDD

After a number of experiments with EDD, sufficient data was obtained to permit detailed analysis of primer annealing. One major question was the number of bases required at the 3' end of the arbitrary primer in determining specificity. From the described

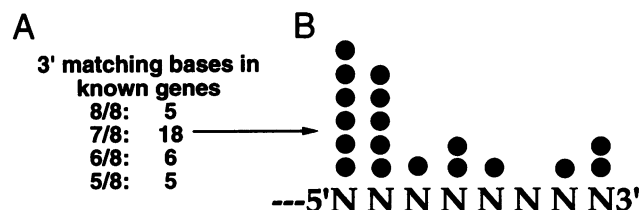


**Figure 1.** Three examples of EDD autoradiographs. The primer combinations are indicated above the gel. Lane 1, BJ cells old, Passage Doubling Level (PDL) 90.3, grown in 0.5% serum (see Materials and Methods for precise growing conditions); lane 2, BJ cells young, PDL 40, grown in 0.5% serum; lane 3, IMR90 old, PDL 54, grown in 0.5% serum; lane 4, IMR90 young, PDL 21.4, grown in 0.5% serum; lane 5, IMR90 old, PDL 53, grown in 10% serum and lane 6, IMR90 young, PDL 27.4 grown in 10% serum.

catalog (see below) and other experiments we collected 34 genes with known sequences. The site for the arbitrary primer annealing was determined by matching gene sequences with primer sequences and band size. The complementarity for the eight bases at the 3' end were determined. Matches with the gene sequence for bases 9–21 (3'–5') in the primer were highly variable and were not considered to contribute to the initial annealing of the primer. Figure 2 summarizes the mismatches in the 3' most eight bases for 34 genes. Clearly, mismatches in the last eight bases were permitted and in some cases more than one mismatch was found. Mismatches occur more frequently toward the 5' end of the primer, as expected. Overall, the data in Figure 2 suggests that a 7 out of 8 match is the typical behavior of a 3' primer in EDD.

#### Variations in efficiency of anchored 3' oligo dT primers

During the course of the experiments, 216 bands were identified as being differentially expressed. The association of these bands with the primer sets that generated them provides an indication of the efficiency of each primer set. It is assumed that if a primer set displays more efficiently, more differential bands will be identified. In other words, the number of differentially identified



**Figure 2.** Analysis of the frequency of mismatches in the 3' most eight bases of the 5' primers used in EDD. (A) Frequency of mismatches detected in 34 genes. (B) Distribution of the position of the mismatch in those sequences that have a single mismatch.

bands reflects the efficiency of the primer set. Analysis of the data indicates that the efficiency of a primer set was mostly determined by the 3' T-rich primer and not by the 5' primers.

The efficiency of the 3' primers was analyzed by collecting data for each 3' primer combined with all twenty 5' primers, resulting in 240 total combinations. Each primer combination was used with RNA from 8 to 11 different fibroblast samples. We found that the number of distinctive bands displayed was dependent on the selected primer pair. A subjective interpretation of the efficiency of the display was made for all 20 arbitrary primers paired with a particular 3' primer (Table 2). The number of identified differential displayed bands for each 3' primer was also determined (Table 2).

**Table 2.** Efficiency of the 3' T-rich primer in EDD

3' End primer	Efficiency	No. of identified EDD bands
CT	poor	6
AT	poor	0
AA	moderate	10
AC	moderate	14
AG	moderate	13
CC	good	13
CG	good	26
CA	good	15
GT	good	24
GG	good	28
GA	good	25
GC	good	42

Gels were judged subjectively as explained in the text (efficiency); the number of identified bands that are expressed differentially is indicated. It is assumed that the number of identified differentially expressed bands is proportional to the total number of bands that can be displayed by each primer pair.

As has been noted before (12), the efficiency varies based on the last two 3' bases of the T-rich primer. As seen in Table 2, primers that were judged to work well in EDD generate overall more differentially displayed genetags. The data presented here and the data from others (12) are in general agreement, although some differences are seen. While primers ending with CA and GT worked more efficiently in our study, primers ending with AC and AG were more efficient in the earlier study (12). It appears that oligonucleotides ending with G or C or having Gs and/or Cs in the last two bases prime more efficiently. Overall, 165 of 240 primer combinations were judged efficient, with almost all inefficient primer combinations being due to the choice of 3' primer.

The dinucleotide CA has been identified as the most common dinucleotide to which poly-A is added during polyadenylation (42). This fact implies that 3' primers having a G residue following the poly-T stretch should generate more cDNA bands than other primers. The data from Table 2 show that 119 bands (24 GT, 28 GG, 25 GA and 42 GC) out of 216 (55%) are generated by such primers, in agreement with the previously observed frequency of 59% (42).

### The use of EDD in generating a catalog

The estimated number of different mRNAs in a typical eukaryotic cell range from 10 000 to 20 000, with most of the messages expressed at low levels (41,43). An average EDD gel in our practice displays ~50 bands in the size range of 100–400 bases. Thus, using 165 interpretable primer combinations, ~8000 EDD bands can be displayed. By increasing the length of a sequencing gel it is possible to increase the number of distinct bands to 150–250 per gel (38), which would increase the total number of bands to 24 000–40 000.

An alternative calculation to determine how the number of primer combinations required to catalog most of the expressed mRNA is based on the finding that, on average, seven of eight bases determines the specificity of the arbitrary primer (see above). For the following calculations, it is assumed that the position of a single mismatch is irrelevant, nor is the occurrence of a perfect match (eight out of eight) included in the calculation. The chance for an 8mer to find a seven out of eight match is  $[(1/4)^7 (\text{matches}) \times 3/4 (\text{mismatch})] \times 8 (\text{positions}) = 1/2731$ . So the chance to detect a match in a region of 300 bases is  $300 \times 1/2731 = 11\%$ . Therefore the chance of not detecting a particular sequence in 300 base region with one 5' primer is 89%. With 20 primers, the chance of detecting a sequence will then be  $1 - (0.89)^{20} = 90\%$ . For a 200 base stretch this percentage will be 78%. Therefore, using 20 5' primers in a cataloging experiment appears sufficient to detect most mRNAs in a population, especially if it is considered that EDD most likely will not detect rare sequences.

### A catalog for young and senescent fibroblasts

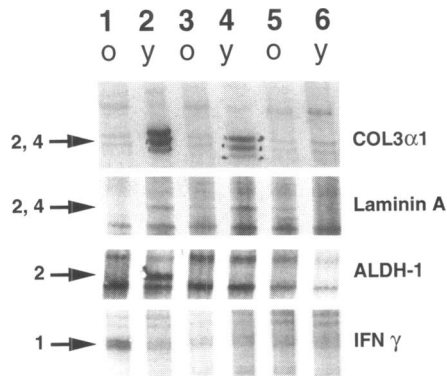
Six fibroblast RNA samples were used to generate our EDD catalog, using all 240 primer combinations described above. These six samples were young and senescent human foreskin fibroblasts (BJ) grown in 0.5% serum for 5 days after passaging and young and senescent human lung fibroblasts (IMR90) grown in 0.5% serum as above or grown in 10% serum for 2 days after passaging. The 0.5% serum conditions are considered quiescent conditions, in which young fibroblasts do not reach confluence. The number of dividing cells is low in these populations as measured by BrdU incorporation or CyclinB staining (generally below 5%, data not shown). The 10% serum conditions are considered mitotic conditions, in which the young fibroblasts have not reached confluency and are actively dividing, as judged by BrdU incorporation or CyclinB staining (data not shown).

After initial inspection of the EDD gels, 168 bands were identified as being differentially displayed. The differential display of four bands is illustrated in Figure 3. Each band was extracted from the acrylamide gel and re-amplified using the same primers (see Materials and Methods). The resulting fragments were separated from the primers on a low melting agarose gel (see Materials and Methods) which also allowed for a verification of the size of the re-amplified band. The band was excised from the gel and the DNA in the gel slice was used directly for DNA sequencing (see Materials and Methods). For 84 bands (50% of total), ~30–40 bases of sequence could be determined accurately without further manipulation. DNA sequence from the remainder suggested that most bands appeared to consist of a mix of more than one specific DNA sequence. This problem has been observed before although it was interpreted as being due to the presence of two primers in the sequencing reaction (35). The data from this study indicate that even after primer removal, ~50% of the bands could not be sequenced directly. Therefore, contamination of identified differential displayed bands with other sequences can be a problem in using the DD technique.

**Table 3.** Summary of differentially expressed EDD bands associated with replicative senescence in HDFs

Cells	Known genes			Novel gene tags	EDD bands	Total EDD bands
Young IMR90	0			3	9	12
Young IMR90/BJ	6	collagen 1, pro $\alpha$ 2	laminin A	10	16	32
		collagen 3, pro $\alpha$ 1 (14 $\times$ )	EST06636			
		collagen 1, pro $\alpha$ 1 (2 $\times$ )	EST07136 (2 $\times$ )			
Young BJ	5	aldehyde dehydrogenase		2	13	20
		glutamate receptor	osteonectin			
		80K-L protein	EST04288			
Old IMR90	5	lipoprotein-associated coagulation inhibitor		6	13	24
		hematopoetic proteoglycan	tPA (3 $\times$ )			
		CD44	HSCDN7 (EST sequence)			
Old IMR90/BJ	6	tPA (2 $\times$ )	(EST) IB568	7	15	28
		TIMP-2	heregulin			
		human tissue factor	mitochondrial RNA			
Old BJ	5	IGF binding protein 5	MnSOD	9	18	32
		PAI-2 (uPAI)	EST00718 (2 $\times$ )			
		interferon gamma (2 $\times$ )				
<b>Total</b>	<b>27</b>			<b>37</b>	<b>84</b>	<b>148</b>

Direct DNA sequencing and data base analysis identified both known genes and novel genetags, while many EDD bands await cloning and sequence analysis (EDD bands).



**Figure 3.** Differentially displayed bands in EDD analysis of young (y) and senescent (o) fibroblasts. The loading order is identical to that in Figure 1. The arrows on the left indicate the differentially displayed band, with the numbers indicating the lane numbers with elevated expression. The sequence of these bands appeared identical to known genes, which are indicated on the right. See Materials and Methods for GenBank accession numbers. The primer sets that were used for detection of the bands were 02 and C for Col3α1, 16 and C for Laminin A, 18 and C for ALDH-1 and 01 and D for IFN  $\gamma$ .

Sequence analysis revealed that about half of the differentially displayed bands, such as those in Figure 3, are identical to known genes and several genetags appear to derive from the same mRNA. Due to the arbitrary selection of the 5' primers, it is expected that some mRNAs will be identified more than once in a screen that covers between 50 and 90% of all mRNAs. A summary of the identified genetags for each cell strain is given in Table 3. Some of these genetags are specific for a growth condition, while others are differentially expressed independent of the growth conditions.

Several known genes were identified more than once. The collagen type 3, pro alpha 1 chain, gene product was identified 14 times as a relatively short band (120–140 bases), identified with several independent 5' primers and several different 3' primers. One band was derived from a poly-A stretch located ~700 bases from the 3' end of the mRNA. A favorable choice of primer pairs combined with the high abundance of this message, accounts for the repeated detection of this message. In some cases, a gene was identified with the same 5' primer in combination with different 3' T-rich primers (not shown). This demonstrates that the two anchored bases in the 3' primer do not confer absolute specificity as has been observed previously (1).

Although the gene expression pattern in the two HDF strains is very similar (Fig. 1), some differences were observed when comparing senescent IMR90 and BJ fibroblasts (Table 3). A number of alterations in expression are shared between the two strains, but others, such as the up regulation of CD44 in senescent lung fibroblasts and the up regulation of Plasminogen Activator Inhibitor 2 (PAI-2) in senescent foreskin fibroblasts, are apparently cell strain specific. With three independent primer pairs, the expression of tissue Plasminogen Activator (tPA) was identified as being elevated for senescent IMR90, but it was identified with two other primer pairs as being elevated in both BJ and IMR90 senescent fibroblasts. These observations indicate that some primer-dependent variability in display may occur during EDD, thereby emphasizing the need for verification of the initial observations by Northern analysis.

## Verification of differential expression

Genetags that could be readily sequenced were used for further analysis of the data generated by EDD. Oligonucleotide probes for Northern analysis were designed that were complementary to many of the known genes listed in Table 3 (see Materials and Methods). While no probes were generated for the EST sequences, five probes were prepared for genes that were previously characterized as being differentially regulated in young and senescent cells [PAI-1 (44), elastin (45), EPC-1 (46), collagenase (47) and urokinase type plasminogen activator (48); see Fig. 4B]. RNA was prepared from both young and senescent IMR90 and BJ fibroblasts, grown in either 0.5 or 10% FBS. These eight RNA samples allow a comprehensive analysis of the expression of genetags identified by EDD. Northern analysis of previously characterized genes with altered expression in young or senescent cells was performed for all five genes and the reported differential expression was confirmed (Fig. 4B). Of the 21 known genes identified by EDD (not including ESTs), Northern analysis confirmed differential expression for 12 genetags (see Fig. 4C), including the four differentially displayed bands from Figure 3, while seven genetags failed to show the predicted differences (data not shown). The hybridization pattern of one probe was non-specific and one gene (mitochondrial RNA) was not analyzed.

Of the 37 novel genetags (Table 3), 31 were recovered after cloning. Two genetags were identified twice in EDD, leaving 29 unique genetags to be tested. The conditions for specific hybridization of probes for novel genetags were first tested in Southern analysis. Genetags are most often generated from the untranslated 3' end of the message, a region rich in repetitive-type sequence. Of the 28 probes that detected specific bands in Southern analysis, five probes did not give a signal in Northern analysis (20  $\mu$ g of total RNA was loaded per lane), 12 probes did not confirm the EDD observations and 11 probes (see Fig. 4) were in agreement with the initial EDD observation. This analysis again demonstrates that about half of the genetags tested confirmed the initial EDD observation.

## DISCUSSION

### EDD

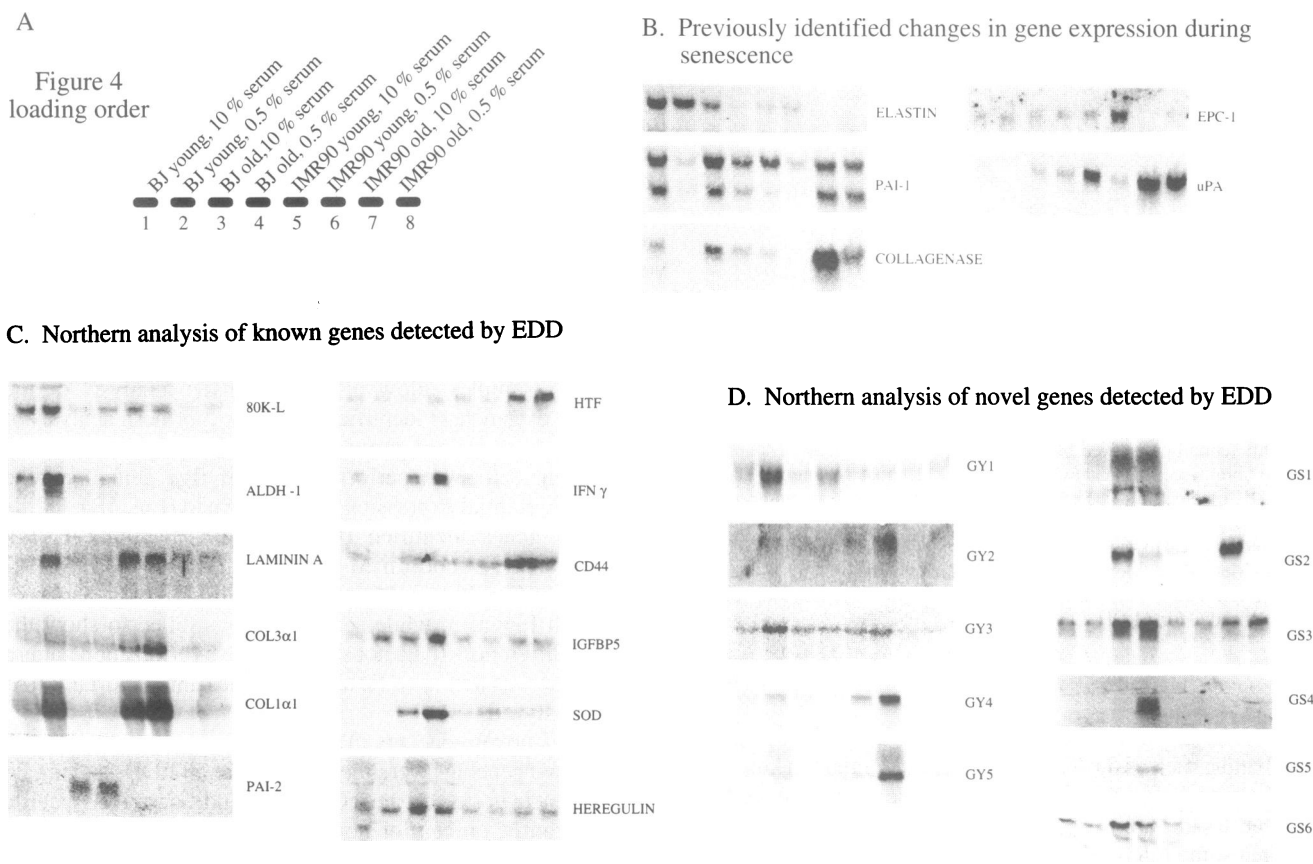
During the process of cataloging genes with altered expression in young or senescent cells, we developed a reproducible method of DD, called EDD. This technique proved to be a reliable tool in discovering differentially expressed genes. We note, however, that all of the known genes found to be differentially expressed by EDD were rather abundant, because they can be detected on a Northern blot that contains 20  $\mu$ g of total RNA per lane. We suspect, therefore, that rare genes may not be efficiently detected by EDD and DD.

### Differentially expressed genes during replicative senescence

A number of genes have been reported to be differentially expressed during replicative senescence (21,25,28,31) and five were selected to demonstrate their differential expression in the IMR90 and BJ fibroblasts used in this study (see Fig. 4).

Several of the known genes identified in this study, such as PAI-2 (49), collagen type 1 pro alpha 1 and collagen type 3 pro alpha 1 (50,51) have been reported to be expressed differentially





**Figure 4.** Northern blots of RNA from young and senescent cells. (A) Loading order (for B, C and D) of the RNA samples. Twenty  $\mu$ g of total RNA was loaded in each lane and loading was verified by ethidium bromide staining of an identical gel (data not shown). The probes used in (B) detect genes that were reported previously to be expressed differentially between young and senescent cells (see text for details). (C) Probes for known genes that were identified by EDD to be expressed differentially; and (D) probes for novel genes that were identified by EDD to be expressed differentially.

in young and old cells and thus serve to validate the EDD technique. Manganese superoxide dismutase (MnSOD), an enzyme that protects cells from oxidative stress by scavenging superoxide radicals in the mitochondria (52), had been found to be up-regulated in senescent foreskin fibroblasts (49,53), similar to our observations (Fig. 4B). Insulin-like growth factor binding protein 5 (IGFBP-5) (54) was found in this study to be abundant in senescent cells, which is notable because IGFBP-3 had been found previously to be up-regulated during senescence and quiescence (34,55,56).

A role has been suggested for interferon gamma (IFN  $\gamma$ ) in the regulation of senescent-specific gene expression in fibroblasts (57). IFN  $\gamma$  increases expression of collagenase and fibronectin and reduces the mitogenic action of growth factors (57). These alterations in expression are very similar to changes in gene expression seen during replicative senescence, which suggests a role for IFN  $\gamma$  in the senescent phenotype (57). Our finding that IFN  $\gamma$  is expressed in senescent foreskin fibroblasts (Fig. 4) correlates well with this proposed role for IFN  $\gamma$ . Other factors that may be involved in altered growth and mitotic regulation were found in this study (Fig. 4), including human tissue factor (58), cell adhesion molecule CD44 (59) and heregulin (60). Heregulin was identified as a specific activator of p185erbB2 and may represent its natural ligand (60). The heregulin probe used in this study detected three transcripts, of 6.5, 2.5 and 1.8 kb, as has

been described previously (60). The differential expression is most pronounced for the 2.5 kb transcript (Fig. 4), but the nature of each of the three different transcripts is not yet clear (60).

Aldehyde dehydrogenase 1 (61), the calmodulin binding protein 80K-L, a substrate for protein kinase C (62) and laminin A (63) are down-regulated during replicative senescence (Fig. 4). Reduced expression of these genes may contribute to the senescent phenotype, although our current understanding of their roles is not clear.

Several known genes, such as those shown in Figure 4B, clearly show altered expression patterns by Northern blot analysis, yet were not identified by EDD. Some of these sequences may be present in the 84 bands that could not be sequenced directly. Cloning and subsequent analysis of these bands will likely identify additional novel and known sequences and will generate a more comprehensive catalog of senescence-related genetags.

For several genetags identified by EDD, we were unable to confirm the suggested differential expression by Northern blot analysis (compare Table 3 and Fig. 4). Two of these genes, tPA and TIMP-2, have previously been described as being up-regulated in senescent cells (21,64), similar to our observations with EDD (Table 3). Work is in progress to determine the reason for the observed discrepancies between EDD, literature and Northern blots.



The initial catalog of 28 differentially expressed genes, verified by Northern analysis in two strains, is the most comprehensive to date for senescent cells. This catalog will be enlarged by the ongoing study of the genetags that could not be sequenced directly and will provide a solid basis for the further study of the senescent phenotype. A better understanding of replicative senescence in turn should lead to novel insights into the role that replicative senescence may play during the aging of an organism (28,31).

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